COMPARISON OF THREE TECHNIQUES FOR DETECTING ENTEROTOXIN A (SEA) IN CLINICALLY RELEVANT *STAPHYLOCOCCUS AUREUS* STRAINS

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COMPARISON OF THREE TECHNIQUES FOR DETECTING ENTEROTOXIN A (SEA) IN CLINICALLY RELEVANT *STAPHYLOCOCCUS AUREUS* STRAINS

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DEDICATION

This thesis is dedicated to my parents, Cary and Beth Harrison, for their love and support throughout the years. I would not have been able to complete this degree without their help.

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ABSTRACT

Thirty-three clinical strains of *Staphylococcus aureus* were screened for the production of staphylococcal enterotoxin A (SEA) protein and/or its gene using three techniques, reversed passive latex agglutination (RPLA), Ouchterlony double diffusion (ODD), and polymerase chain reaction (PCR). Of the isolates, two produced detectable levels of SEA (6%), while 22 (64.7%) harbored the gene for SEA. These results indicate that clinical *S. aureus* isolates commonly have the potential of producing SEA. ODD testing revealed promise for using it to detect prolific producers of enterotoxins. RPLA was shown to detect enterotoxins with specificity and accuracy. PCR revealed that although clinical strains frequently have the *sea* gene, they often do not produce SEA. Further studies should examine the presence of other staphylococcal enterotoxin genes and products. Growth conditions should also be evaluated to determine the ideal environments for enterotoxin production.

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INTRODUCTION

Staphylococcus was first described in 1882 by Sir Alexander Ogston. *Staphylococcus* is a genus characterized by gram-positive, nonmotile, spherical (coccus) bacteria that form clusters resembling grape bundles (14, 21). The bacteria are commonly found on the skin of animals and in the nasal passages. They are halotolerant and are facultative anaerobes and therefore can survive with or without oxygen (21). Some species of *Staphylococcus* are opportunistic pathogens and can be resistant to many antibiotics. These bacteria are commonly associated with nosocomial, i.e. hospital acquired, infections (15, 21, 22).

The species of greatest concern is *Staphylococcus aureus*. *S. aureus* is an opportunistic pathogen that typically invades broken skin. The bacteria have many properties that allow them to avoid the immune system and increase transmission and infection rates. Some *S. aureus* strains are problematic because of the production of toxins, called enterotoxins, causing food poisoning or toxic shock syndrome. Staphylococcal enterotoxins (SEs) can cause acute food-borne illness characterized by a short incubation period (3 to 5 h) with nausea, vomiting, abdominal cramping, and weakness. Some fatalities have been recorded, but these are rare and usually occur in the young, elderly, or immunocompromised patients (19, 33). There are 24 different SEs that have been described, but the toxicity has not been determined for all. SEA, SEB, SEC, SED, and SEE are considered the classical SEs that have been studied and are better understood than the newly described SEs (SEG-SEU) (20, 28, 29). *S. aureus* is one of the leading causes of food-borne illness worldwide, caused primarily by either the production of SEA or SEB. There is confusion as to whether SEA or

SEB is the more predominant cause of food poisoning (2, 8, 9, 23). A wide variety of foods support growth of *S. aureus* and are ideal for enterotoxin production including: milk, raw milk, meat, meat products, dairy products, and ready-to-eat food. Foods that are protein-rich, have a neutral pH, and are extensively handled are more likely to be contaminated with and support the growth of *S. aureus* thus leading to enterotoxin production (2, 9, 10, 33). SEA and SEB can cause disease in very small amounts, 100-200 ng/ml or 10-20 ng/ml respectively (6, 7, 8, 9).

Since staphylococcal enterotoxins can cause disease in such low concentrations, rapid and sensitive detection is needed in order to diagnose the illness or identify the contaminated food correctly. Many methods have been developed in order to detect the toxins quickly with specificity and sensitivity which include: an immunoassay single diffusion tube test, polymerase chain reaction (PCR), an enzyme-linked immunosorbent assay (ELISA), a reversed passive latex agglutination assay (RPLA), and the Ouchterlony double diffusion method (ODD) (1, 10, 13, 29). Several factors must be considered when choosing a method for enterotoxin detection, such as sensitivity, specificity, reproducibility, cost, labor, rapidity, convenience, and the number of samples.

The ELISA method is commonly used because reagents are commercially available and sensitivity of the test is reliable. Clarisse et al. used a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) to detect SEA in dairy products and different foods. The authors found that this method was quick, easy, and could be used for routine food product SEA quantification (9). However, the cost of the test is high and detection of SEs is currently limited to the classical types, so therefore it is not an ideal method for hospitals or the food industries to use.

The RPLA test is the method that continues to be superior (23,28,33) when compared to other methods for detecting enterotoxins. The SET-RPLA kit is commercially available and is sensitive and specific for the classic types of SEs. The incubation period is also less than 24 hours and the results can be viewed with the unaided eye, unlike the ELISA. In 1988, Fijukawa and Igarashi developed a method for a rapid RPLA test with high-density latex particles that uses only a 3 h incubation time, but further testing has not been presented. Their data suggested that their method was highly specific and sensitive for detection of SEs, similar to the commercial test kit. RPLA and ELISA are commonly compared for their specificity and sensitivity, in which they reproduce similar results (13). Schumacher-Perdreau et al. compared the two tests when detecting SEB and found similar results with both tests. They also carried out PCR and found the *seb* gene in strains that were positive for SEB with the ELISA and RPLA test (28). In 2004, Di Pinto et al. compared RPLA and immunoblotting methods for SE detection. Their results showed that SET-RPLA is quick, sensitive, and specific for the enterotoxins tested (SEA-SED) and recommended that it continue to be used to test numerous food samples for routine monitoring (10). Even though RPLA has been shown to be adequate for identification of enterotoxins, there are still some limitations related to cross reactions that lead to false positives (29).

PCR is commonly used for enterotoxin gene detection, or for confirmation of results from other tests. PCR has been performed with RPLA but the test is limited to the presence of the gene for the enterotoxin, not the production of the enterotoxin itself. In 2007, Morandi et al. used PCR to detect *sea, sec, sed, seg, seh, sei, sej,* and *sel* and followed with RPLA for detection of SEA-SEE (23). Over half of the *S. aureus* strains tested had *se* genes, the majority containing the classical *se* genes (*sea-sed*). Only 20 of the 75 strains positive for

toxin genes contained just one type, the others contained two or more *se* genes. There was an 80% correspondence with the SE production shown by SET-RPLA and the *se* detected by PCR in all *S. aureus* isolates analyzed (23). In cases where non-correspondence was observed, usually *se* genes were detected but the corresponding SE was not. This could be because the gene was not expressed or it was expressed at levels too low to be detected by SET-RPLA. In only 3 strains, RPLA showed SE production, while PCR showed no corresponding *se* detection (23). One reason for this result could be mutation in the gene sequence or cross reaction in the RPLA testing. Zouharova and Rysanek also compared multiplex PCR and RPLA (33). In their study, PCR detected more *se* genes than RPLA detected SE production. PCR was used to identify the classical *se* genes (*sea-see*) and the new *se* genes (*seg-sel*), while RPLA could only detect SEA-SED. In all strains that revealed *seb* and *sed*, SEB and SED were discovered, but in every strain where *sea* was detected, SEA was not present according to the RPLA (33). Despite the discrepancies, the authors suggest that RPLA should be the 'gold standard' for SE detection. Other authors have used PCR to compare the effectiveness of enterotoxin detection in other techniques such as pulsed-field gel electrophoresis (PFGE) and the lateral flow assay (also known as immunochromatographic procedure)(2, 8). PCR is a good way to detect the genes for the enterotoxins, but it is limited to the gene and not the production of the toxin.

The ODD test was developed by Orjan Ouchterlony, and many versions and modifications have been performed (24). Double diffusion refers to the fact that both an antigen and an antibody are diffusing through a gel. When a reaction between the two take place, a line of white precipitate forms in the gel giving a positive reaction. This test was developed to determine the concentration of antigen or antibody needed for detection or to

match an antigen with its appropriate antibody. A modification of the ODD test has been developed by Casman; this method places the antiserum in a centrally located well in a shallow layer of agar in a petri dish and antigens are added to peripheral wells (5). Adesiyun et al. compared Casman's modified ODD test with RPLA test and ELISA (1). Staphylococcal strains were taken from humans and other animals and cultured on Baird-Parker agar. The supernatant of each strain of *S. aureus* was simultaneously screened for enterotoxin production by the modified ODD test, then the RPLA test, followed by the ELISA, using supernatant from the same growth. After screening the more than 1,000 strains, RPLA and ELISA detected significantly more enterotoxins than the modified ODD tests, with no significant difference between the RPLA test and ELISA (1). The detection limit for the SEs ranged depending on what test was being used. The modified ODD test could only detect concentrations of 5µg/mL, while the RPLA test detected a range from 0.5 to 2.25 ng/mL. The ELISA detection range varied based on the enterotoxin type being detected, but for all SEs the detection concentration was much lower than the ranges reported for the ODD test (1). The sensitivity of the ODD test has been shown to be limited to detection of microgram quantities of toxin as opposed to nanogram quantities detectible by the other tests. Based on their results, the authors concluded that RPLA testing was preferable to the other two methods due to convenience, sensitivity, specificity, ease, and rapid nature of the test (1).

Kimberly Dybdahl, a previous graduate student at Angelo State University (2003), conducted multiple experiments using a modified Ouchterlony double diffusion method with the goal of developing a time saving and economically feasible test for detecting SEA producing strains. Using a strain of *Staphylococcus aureus* known to produce SEA, she compared several different media under various conditions to determine optimal conditions

for enterotoxin detection. Considering all the results, Dybdahl recommended the use of Todd Hewitt Broth supplemented with 1% agar (less than 2.5mm thick). A 35°C growth phase and a room temperature precipitation phase using 30 µl of the antibody and control toxin were also recommended for the maximum likelihood of enterotoxin detection. This study had limitations due to the amount of time needed before being able to read the results and the lack of sensitivity when compared with other tests such as RPLA test and ELISA (11). Nevertheless, she found that the method was simple, inexpensive, and useful for screening strains of *S. aureus* that are capable of producing large amounts of SE as well as allowing the investigator to visualize cross-reactions in the gel.

The goal of this thesis research was to compare the sensitivity and specificity between RPLA and the ODD tests for detection of SEA in previously identified *S. aureus* strains isolated from infected patients at a local hospital. PCR was used to confirm the presence of *sea* genes in these strains. By comparing results among these methods I tested the hypothesis that RPLA and ODD tests have high specificity for detecting SEA and that no cross-reactions or contamination will occur in these tests.

In addition, the outcomes of this study allowed me to address several questions: 1) Do clinical isolates of *S. aureus* carry SEA more often than isolates from the community? 2) Do clinical isolates produce microgram quantities of SEA as revealed by ODD? 3) Can ODD reveal cross-reactions that might complicate interpretations of RPLA? and 4) How frequently was the SEA gene present but not detected in the RPLA test?

MATERIALS AND METHODS

Sample Characteristics: Thirty-three *S. aureus* samples were received from a local hospital from patients diagnosed with staphylococcal infections in 2010. Samples were taken from the site of infection and susceptibility/resistance to antibiotics was tested at the hospital. All data and cultures, excluding names of the patient, were given to Angelo State University for experimental use. Very little is known about the infection from which the samples were obtained except the site of infection (Table 1). Some of the samples were found to be resistant to up to 9 different antibiotics (Table 1). Most of the samples were resistant to ampicillin, penicillin, or erythromycin (Table 2).

Table 1. Characteristics of clinical *Staphylococcus aureus* isolates used for testing.

Pathogen ID #	Age of Patient	Source	# of Antibiotics Resistant to
17	21	neck wound	6
35	17	thigh wound	6
67	40	wound	0
71	28	wound	1
146	38	rt thigh wound	6
151	20	wound	8[a]
158	65	wound	0
171	24	jaw wound	0
173	27	wound	8
226	86	wound	4
253	34	wound	7[a]
278	29	wound	6
301	n/a	wound	5
342	n/a	wound	5
357	40	miscellaneous	0
395	59	misc/groin	0
410	70	knee	1
436	58	wound	8[a]
437	45	wound	6
476	43	wound	1
497	77	sputum	1
571	61	misc/groin	0
636	63	wound	8[a]
638	82	sputum	9[a]
655	n/a	misc/bone	0
669	96	wound	8
670	96	ear	8
672	35	wound/scrotum	1
767	35	misc/scrotum	1
897	66	leg wound	9
972	1	groin wound	6
973	63	abdominal wound	6
982	63	wound	8[a]

a - Isolate was considered intermediate to at least one drug

Table 2. Antibiotic resistance observed with the clinical isolates of *S. aureus*

Antibiotic	Total Number of Isolates Resistant	Percentage of Isolates Resistance (%)
Amox/K Clav[a]	17	57
Amp/Sulbactam	19	58
Ampicillin	31[b]	0
Cefazolin	19	58
Ceftriaxone	19	58
Ciprofloxacin	9	27
Clindamycin	4	12
Erythromycin	26	79
Gatifloxacin	1	3
Gentamicin[a]	0	0
Levofloxacin	8	24
Linexolid	0	0
Oxacillin	19	58
Penicillin	31[b]	0
Rifampin	0	0
Synercid	0	0
Tertracycline	2	6
Trimeth/Sulfa	0	0
Vancomycin	0	0

a - Not all samples were tested with this drug

b - Beta-lactamase positive

RPLA (Staphylococcal Enterotoxin Test Kit, OXOID; Basingstoke, Hampshire, England): Thirty-four centrifuge tubes with 10 mL of Todd Hewitt broth (Acumedia, Lansing, Michigan) were inoculated with 34 different clinical isolates of *S. aureus* including a positive American Type Culture Collection control strain positive for SEA. The tubes were incubated for two days at 35°C and then vortexed before being placed in a clinical centrifuge for 30 minutes to separate the supernatant and the precipitant at 2000 RPM. Following manufacturer instructions, 25 µL of diluent was added to each well of a 96-well plate used for testing. The first well for each column was then filled with aliquots of the supernatant (25 µL) of each culture tube and double dilutions were performed until four wells were used. Antibody for SEA (25 µL) was added to each well containing the diluted supernatant, with mixing by pumping the micropipette thrice and throwing away each tip between transfers to prevent contamination. The plates were incubated at room temperature and read the following day. Negative reactions were evidenced by a button of growth at the bottom of the well and a positive reaction was evidenced by a flocculent turbidity in the well.

Using the same isolates, 34 centrifuge tubes with 10 mL of Tryptic Soy broth (Acumedia, Landing, Michigan) were inoculated. The tubes were incubated at 35°C, vortexed and placed in the centrifuge, the same as the prior test. For this test, no dilutions were performed. Antibody and supernatant (25 µL of each) were added to each well and results were observed the following day.

Modified ODD Test: All strains were tested for SEA using the following model originally described by Dybdahl (11): 1) all strains were streaked within a 2.54 cm by 1.27 cm rectangle on 1% agar (Difco supplemented) plates of Todd Hewitt Broth; 2) two wells were cut parallel to one another and located 9 mm from the rectangle; 3) the wells were cut 6 mm

from one another; 4) one well contained 30 µl of the appropriate control SE, and the other well contained 30 µl of the anti-*S. aureus* enterotoxin A antibody (Ab); 5) wells were not punched into the plates until immediately before SE and Ab were added; 6) only plates with agar depths ranging from 2.5 mm to 5.0 mm were used and; 7) each appropriate Ab well contained undiluted antiserum, and each control well contained 100 µg/ml of SEA enterotoxin. After the plates were inoculated with the various strains, they were incubated for 24 h at 35°C, prior to the wells being cut and antiserum and control added. After the addition of these two, the plates were observed at room temperature every day. Precipitation results were recorded every day for 3 consecutive days following the introduction of the SE control.

PCR: PCR reactions followed the methods used by Zouharova and Rysanek (33), but for only the *sea* gene. The *nuc* gene was used as an internal control of DNA specificity and to verify the PCR reaction process. Water in place of the template DNA acted as a negative control for each reaction. To isolate the DNA, colonies of pure bacteria were resuspended in 50 µl of sterile distilled water and incubated for 10 min at 100°C and centrifuged for 1 min at 16,000 g at 4°C. The supernatant was used as the template DNA for the PCR reaction. The reaction mixture consisted of 20 µl containing 2 µl of supernatant with template DNA, 1X QIAGEN PCR Buffer, 2.5 U *Taq* DNA polymerase (Qiagen *Taq* PCR Core Kit), 200 µM of each dNTP (Qiagen *Taq* PCR Core Kit), 4.5 mM $MgCl_2$, 0.13 µM of each primer (AlphaDNA, see Table 3), and sterile distilled water used to attain a final volume of 20 µl. For amplification the following process was used: initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 62°C for 45 s and extension at 72°C for 1 min 45 s, followed by a final extension for 8 min at 72°C. The amplified PCR products were distinguished by standard gel electrophoresis in a 2% agarose

gel and visualized after staining with ethidium bromide using a transilluminator. A middle range ladder (Fermentas FastRuler Ladder), was used for markers and the gel electrophoresis ran for 55 min at 100 V (33).

Table 3. Primers used for detection of staphylococcal enterotoxin A (*sea*) genes and thermonuclease (*nuc*) gene

Primer name	Oligonucleotide sequence (5'→3')	Target gene	Product (bp)
NUC-f	AGT TCA GCA AAT GCA TCA CA	*nuc*	400
NUC-r	TAG CCA AGC CTT GAC GAA CT	*nuc*	400
SEA-f	TAA GGA GGT GCC TAT GG	*sea*	180
SEA-r	CAT CGA AAC CAG CCA AAG TT	*sea*	180

RESULTS

RPLA: Preliminary testing for SEA-SED revealed variable results with the clinical isolates. The tests showed that in addition to SEA, other enterotoxins were present in the strains tested (data not shown). RPLA testing was performed for all clinically isolated samples using Todd Hewitt broth and Tryptic Soy broth for SEA only. Reactions were classified as positive if any turbidity was present in the well and negative if a button of inoculum appeared at the bottom of the well with clarity in the fluid of the well (Figure 1). Only three (8.8%) of the 34 tested isolates were positive for both tests (Table 4). The American Type Culture, known as Control from hence forth, isolate 669 and isolate 670 produced enough SEA to be detected at all dilution levels when cultured in Todd Hewitt broth. When inoculated in Tryptic Soy broth, the same three isolates produced enough enterotoxin to be detected. All other isolates were negative for all dilutions and culture conditions.

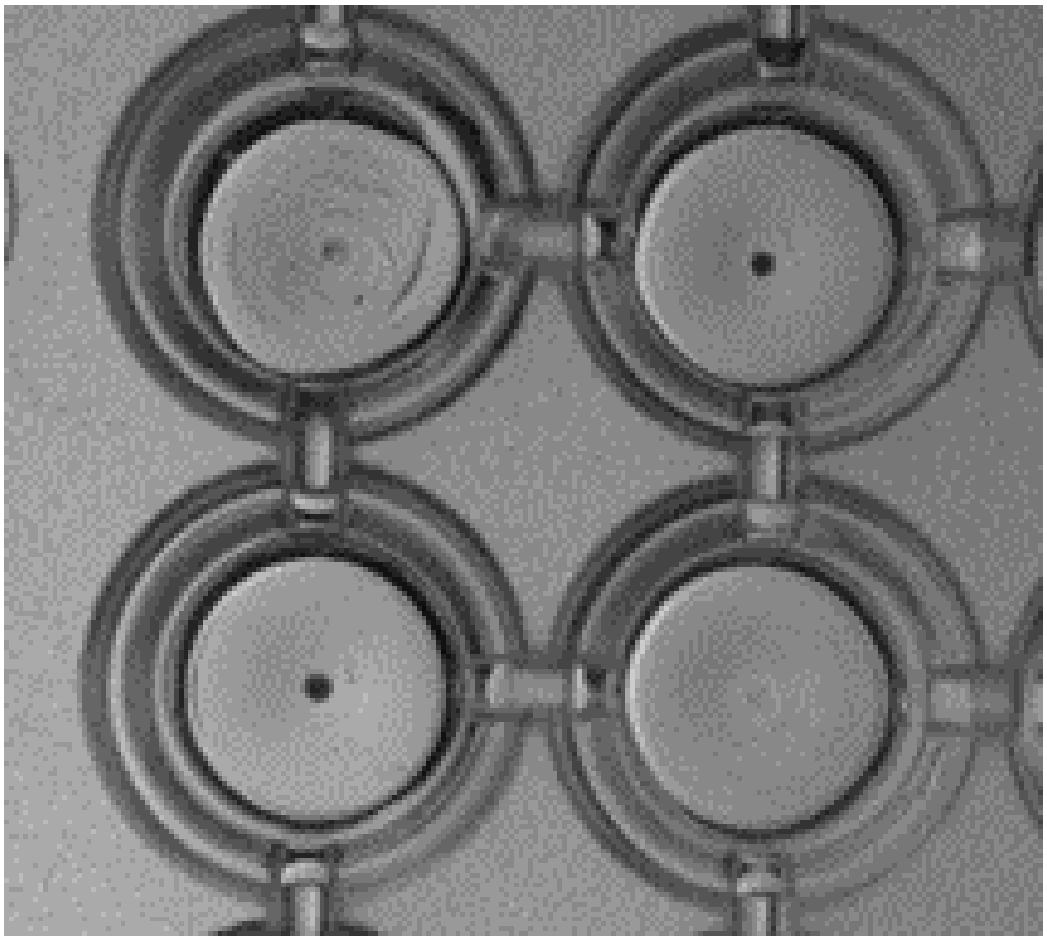

Figure 1. An example of results seen in a RPLA test with *S. aureus*. The top left and bottom right represents a positive. A negative result is shown in the top right and bottom left corners.

ODD: Samples that were not positive during the RPLA test also were negative with the ODD test for SEA. The same three samples produced enough enterotoxin to be detected by the ODD tests (Table 4). The test was repeated twice, and each time the Control, 669, and 670 were the only samples producing enough SEA to be identified. The precipitation line for all samples was a clean curve (Figure 2) signifying that cross reactions between enterotoxins were not occurring in these samples.

Figure 2. Results of ODD tests using clinical isolates of *S. aureus*. Samples 669, 670, and Control (SEA) all show a positive result, while sample 158 shows a negative.

PCR: All samples were screened for *sea* and *nuc* (as an internal control). In total, 22 (64.7%) of the 34 samples had the *sea* gene present. Samples that produced SEA according to RPLA and ODD were in accordance to results seen with PCR. Nineteen of the 34 samples that did not produce SEA according to the two previous tests had presence of *sea* (Table 4). Twelve (35.3%) samples were negative for SEA product and gene in all three tests.

Table 4. Summary of all results for clinically isolated *S. aureus* using RPLA, modified ODD, and PCR.

Pathogen ID	RPLA-THB	RPLA-TSB	ODD	*sea* Present
17	-	-	-	-
35	-	-	-	+
67	-	-	-	-
71	-	-	-	+
146	-	-	-	-
151	-	-	-	+
158	-	-	-	-
171	-	-	-	-
173	-	-	-	+
226	-	-	-	+
253	-	-	-	-
278	-	-	-	+
301	-	-	-	-
342	-	-	-	+
357	-	-	-	-
395	-	-	-	+
410	-	-	-	+
436	-	-	-	-
437	-	-	-	+
476	-	-	-	+
497	-	-	-	+
571	-	-	-	+
636	-	-	-	+
638	-	-	-	-
655	-	-	-	+
669	++++	+	+	+
670	++++	+	+	+
672	-	-	-	+
767	-	-	-	+
897	-	-	-	-
972	-	-	-	+
973	-	-	-	-
982	-	-	-	+
Control	++++	+	+	+

++++: Positive with all four dilutions

DISCUSSION

Increasingly, antibiotic resistance has been associated with *S. aureus*. Of the 34 isolates of *S. aureus* tested in this study, only seven were susceptible to all antibiotics tested, excluding ampicillin and penicillin. Two isolates, 226 and 655, were susceptible to ampicillin and penicillin, both isolates containing *sea*. There was no trend in the pattern of antibiotic resistance seen between samples with *sea* and samples without. For example, many samples were resistant to erythromycin, regardless of *sea* presence or absence, while all were susceptible to vancomycin. Susceptibility to vancomycin has been seen in other studies. Sabouni et al. discovered susceptibility to vancomycin in all samples taken from hospital patients (27). Nevertheless, most studies have reported no correlation of enterotoxin production or gene presence with antibiotic resistance. Likewise, the present study did not reveal any patterns between an isolate's resistance to antibiotics and its enterotoxin characteristics.

The production of SEs by *S. aureus* has been found to be dependent upon strain. Usually, *S. aureus* strains capable of producing SEA are common in humans, as seen with this study. This study focused on clinical isolates of *S. aureus*, however others have looked at isolates from the community as well. Verkaik et al. surveyed over 200 patients with *S. aureus* infections, some they considered community-acquired and others considered hospital-acquired. They found no significant difference in the number of enterotoxin genes in isolates between the two categories of infection; though *sea* was seen to have a significant association with bacteremia (31). SEA and SEB has also been associated with septicaemia (17). Fueyo observed SE production in isolates from healthy humans and from food products. SEA-producing isolates were most common in humans, while food products frequently had SEC

or SED-producing isolates (12). Bania et al. isolated 80 samples from the nasal region of patients with upper respiratory infections of which 61% had enterotoxin genes present, with *sec* being the most common. In most isolates, it was common to find multiple enterotoxin genes (3). Another study compared clinical blood cultures and nasal swabs from healthy carriers and found no difference in enterotoxin production. However, the authors saw a high frequency of SEC and SEB (25). Uemura et al. surveyed *S. aureus* isolates from the nose and throat of healthy persons and found that SEB was the most common enterotoxin detected (30). When testing infants diagnosed with sudden infant death syndrome for *S. aureus*, Highet et al. found that *sea* and *see* were the most frequently found of the classical *se* genes (16). All of these studies allow us to answer the question: Do clinical isolates of *S. aureus* carry SEA more often than isolates from the community? We see that more often than not, *sea* is associated with strains that cause infection leading to hospitalization in humans, but it is evident that disease-causing strains can also carry other enterotoxin genes. Enterotoxins other than SEA are commonly associated with *S. aureus* strains isolated from healthy individuals.

RPLA is considered the "gold standard" for use in detecting enterotoxins in *S. aureus* strains (33). The commercially available forms of these tests are limited to the classical SE's and some studies have alluded to there being cross reactions with the antibodies and enterotoxins not yet discovered. In this study, SEA production was found by RPLA in samples that also had *sea* presence according to PCR. However, many of the isolates that had *sea* apparently did not produce enough SEA to be detected by the RPLA test. Janstova et al. observed that storage temperature (15°C vs 22°C) and nutrients can influence the production of enterotoxins (18), so this study used two different broths to culture the bacteria prior to

CONCLUSION

The results of this study indicate that clinical isolates often harbor *sea*, but the production of the enterotoxin is detected far less commonly. By evidence of the ODD test, isolates do not commonly produce enterotoxins in high quantities, but the method used in the present study showed promise as a way to detect prolific producers of enterotoxins. RPLA can be used to detect enterotoxins with specificity and accuracy. PCR reveals that clinical strains frequently have *sea*, but do not necessarily produce SEA. Further studies should include examination of the presence of other staphylococcal enterotoxin genes and products in these samples. Other growth conditions should be evaluated in determining ideal environments for enterotoxin production, especially for strains that may be stored prior to testing.

testing for SEA. The nutrient variation had no apparent effect on the outcome of these RPLA tests. Though this study did not detect discrepancies between RPLA and PCR, it is not uncommon for this to occur. Many studies have cited SE production as detected by RPLA, and then failed to detect *se* with PCR (23). This study revealed the presence of *sea* in over half of the isolates, but SEA in a fractional number. This difference likely reveals the variation in the quantities produced by various strains of *S. aureus*. Rosengren et al. observed that changes in pH and temperature affected the production of SEA over time (26). Also, different strains could respond to stress by producing more enterotoxins (32). Nonetheless, RPLA is still considered the gold standard due to its experimental simplicity, sensitivity, and rapidity of results.

ODD testing is desirable for determining whether cross-reactions are present in the tested samples. Nevertheless, the quantity of toxin needed for detection is much higher than other tests developed, such as RPLA and ELISA. According to Adesiyun, the detection limit for ODD testing is 5 µg/mL, while the RPLA and ELISA detection limit is 0.5-2.25 ng/mL (1). The present research showed that the samples with detectable amounts of SEA produced enough to be detected by ODD. Since there was a clear precipitation curve and not precipitation lines that crossed with the control toxin and antibody in all three samples, no cross reaction was present in these samples or at least visible at these higher concentrations. Varying amounts of SEs have been shown to be produced by different *S. aureus* strains in other studies, some of which would not be detected by the ODD test itself. SEA has been detected as low as 64 pg/mL in skim milk and nanogram quantities were discovered in *S. aureus* strains isolated from cheeses (9). Although the clinical samples were not tested for quantity of enterotoxin produced, one can speculate that nanogram quantities are more

commonly produced than micrograms. Nevertheless it is reasonable to think that isolates with more prolific production of enterotoxins might be more likely to harm the human body and therefore the ODD technique might serve as a quick, simple, and inexpensive method to detect such strains.

From this project, it was shown that the gene responsible for SE production is not always expressed or is not used to produce enough enterotoxin to be detected by RPLA tests in clinically associated strains of *S. aureus*. Over half of the samples had *sea*, but only 8.8% had evidence of the production of SEA. This disagreement is seen in other studies as well. Zouharova and Ryanek also reported a higher frequency of *se* genes with fewer occurrences of SE presence (33). However, in a study conducted by Zschock et al., a good correlation between ELISA, RPLA, and PCR was found in samples that were isolated from the mammary secretions of cows (33). Another study compared *se* and SE production from several different sources, including bovine, ovine, and human, and 100% correlation between gene and production was observed (4). Based on these conflicting observations, it is not possible to conclude with confidence the correlation between having the gene and expressing it. *S. aureus* strains have previously been shown to produce different levels of SEs under various physiological and environmental conditions (26, 32); therefore, PCR should not be used solely to identify the presence of classical SEs, due to the unpredictability of the expression of the gene.

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